Characterization of the Anti-BP180 Autoantibody Reactivity Profile and Epitope Mapping in Bullous Pemphigoid Patients[1]

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Bullous pemphigoid is a subepidermal bullous disease of skin and mucosae associated with autoantibodies to BP180. To characterize the humoral response to BP180, we generated a random BP180 epitope library displayed on λ bacteriophage. After validation of the library by epitope mapping of three BP180-specific monoclonal antibodies, 15 novel or known BP180 epitopes were identified using 10 bullous pemphigoid serum samples. Fifty-seven bullous pemphigoid and 81 control sera were then assayed against the selected epitopes. Thirty-one out of 57 (54%) bullous pemphigoid sera reacted with at least an additional antigenic site other than the NC16A, within the extracellular (37%) and intracellular (28%) domains of BP180. In addition, the reactivity with extracellular epitopes of BP180 contained within the residue stretches 508–541 and 1331–1404 appeared to be related to the presence of both skin and mucosal involvement. Finally, a preliminary analysis of the epitope pattern in the disease course indicated that bullous pemphigoid patients exhibit a specific reactivity pattern, and that binding to intracellular epitopes of BP180, in addition to NC16A, may be detectable at an early clinical stage. Our findings provide novel insights into the pathophysiology of bullous pemphigoid and show the potential of the utilized approach as a tool for a rapid diagnosis of bullous pemphigoid patients and their management.

Key words: autoimmunity/BP180/epitope spreading/hemidesmosome/phage display.
J Invest Dermatol 122:103–110, 2004

Bullous pemphigoid (BP) is an autoimmune subepidermal bullous disease of the skin and mucosae that typically affects the elderly. BP is associated with tissue-bound and circulating IgG autoantibodies directed against the basal membrane zone (BMZ) of stratified epithelia (Liu and Diaz, 2001). Patient autoantibodies react with BP230 and BP180 (Stanley *et al*, 1988; Diaz *et al*, 1990), two components of the junctional adhesion complex called hemidesmosome. BP230 (also called BPAG1) is a cytoplasmic protein implicated in the organization of the keratin filament network. In contrast, BP180 (BP antigen 2 or type XVII collagen) is a transmembrane protein that consists of a globular cytoplasmic domain and a large extracellular region containing 15 collagenous domains that lead to homotrimer formation (Giudice *et al*, 1992; Hirako *et al*, 1996; Schäcke *et al*, 1998). This protein contributes to the assembly and stabilization of hemidesmosomes (Hopkinson *et al*, 1995; Borradori *et al*, 1997). Mutations in the BP180 gene underlie the non-Herlitz variant of junctional epidermolysis bullosa, an inherited blistering disorder characterized by skin fragility and defective dermal–epidermal adhesion, an observation attesting to the importance of BP180 in maintenance of skin integrity (McGrath *et al*, 1995).

The vast majority of BP patients possess IgG autoantibodies directed against an immunodominant region mapped in the membrane-proximal noncollagenous region of the BP180 ectodomain, the NC16A domain (Zillikens *et al*, 1997a). Additional antigenic reactive sites, however, located both in the intracellular domain (ICD) and extracellular domain (ECD) of BP180, have been recently described (Murakami *et al*, 1998; Nakatani *et al*, 1998; Egan *et al*, 1999, 2001; Nie and Hashimoto, 1999; Perriard *et al*, 1999; Schumann *et al*, 2000). Several observations indicate that autoantibodies to BP180 are pathogenic. In particular (1) the passive transfer of rabbit IgG antibodies, raised against an extracellular region of murine BP180 homologous with the human immunodominant NC16A domain, into neonatal mice induces a blistering disorder mimicking BP (Liu *et al*, 1993); (2) in gestational pemphigoid, a disease closely related to BP occurring during pregnancy, the transplacentar transfer of anti-BP180 autoantibodies from the mother into the neonate can cause a transient bullous eruption (Jordon *et al*, 1976); and finally (3) the level of autoantibodies against BP180 appears to be related to

Abbreviations: AA, amino acids; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BP, bullous pemphigoid; DIF, direct immunofluorescence; ECD, extracellular domain; GST, glutathione S-transferase; ICD, intracellular domain; IIF, indirect immunofluorescence; NBT, nitroblue tetrazolium; NHI, normal healthy individuals.

[1]Tables 1, 2, 3 and 5 can be found at http://www.blackwellpublishing.com/products/journals/suppmat/jid/jid22126/jid22126sm.htm

the activity and extent of the disease (Haase *et al*, 1998; Schmidt *et al*, 2000; Amo *et al*, 2001; Hofmann *et al*, 2002; Kobayashi *et al*, 2002).

Based on these and other findings (Hall *et al*, 1993) it is thought that autoantibodies to the ECD of BP180 are pathogenically critical, whereas development of antibodies against intracellular antigenic determinants on BP230 and on BP180 is a secondary event in the context of an "epitope spreading phenomenon" (Chan *et al*, 1998). A comprehensive characterization of the antigenic determinants of BP180 recognized by BP autoantibodies, however, has not been carried out so far. Furthermore, no data are available about the pattern of reactivity against different epitopes of BP180 in the disease course. A distinct pattern of reactivity is likely to be critical for both the initiation and perpetuation of the disease and may result in a distinct clinical phenotype (Hofmann *et al*, 2002).

The phage display technology is based on the construction of molecular libraries that can be searched by affinity selection with a target molecule. The general limitation of a prokaryotic system for epitope mapping studies is that post-translational modifications such as hydroxylation and glycosylation do not occur. Nevertheless, this technology has recently been successfully employed to identify disease-specific antigens of various autoimmune disorders such as Sjögren's syndrome and vitiligo (Niwa *et al*, 2000; Kemp *et al*, 2002).

In this study, to gain better insight into the pathophysiology of BP, we have prepared a BP180 random epitope library on λ bacteriophage to identify disease-specific epitopes on BP180. We characterized, in addition to known antigenic reactive sites, several novel epitopes located within both the ICD and ECD of BP180 that are significantly recognized by a large panel of BP patient sera. Furthermore, the reactivity profile in BP patients appeared to relate to a distinct clinical phenotype. Finally, we provide preliminary evidence that reactivity with intracellular epitopes of BP180, in addition to that with NC16A, may be detectable in an early stage of the disease.

Discussion

In this study we have utilized the λ phage display technology for the analysis of the humoral response to BP180 in BP, the most frequent subepidermal autoimmmune disorder of the skin. By testing a representative BP180 random epitope library we have been able (1) to precisely characterize the epitopes recognized by three anti-BP180-specific MoAb; (2) to finely map novel antigenic regions within both the ICD and ECD of BP180 that are targeted with significant frequency by a large panel of BP serum samples; (3) to correlate reactivity with specific extracellular epitopes of BP180 with a distinct clinical phenotype. Finally, our findings indicate that in the course of the disease patients exhibit a distinct epitope pattern and that reactivity against various intracellular epitopes, in addition to that against NC16A, can be observed even in the early stage of the disease.

We first validated our library by mapping the epitopes recognized by three BP180-specific MoAb: 1A8c, 233, and 1D1. In extension to previous studies, our approach allowed us to substantially narrow the location of the epitopes for these MoAb (Borradori *et al*, 1997; Hirako *et al*, 1998). These findings will prove useful for future functional studies of BP180, such as cell adhesion or hemidesmosome assembly inhibition assays.

By using the generated random BP180 epitope λ library, we have been able to identify and further map known as well as novel antigenic primary sequences within both the ICD and ECD of BP180. First, in line with previous findings, 60% of the 57 tested BP serum samples bound to an epitope spanning residues 508–541, located within the immunodominant NC16A domain (Giudice *et al*, 1994). Recent studies have shown that the majority of BP serum samples recognize a similar region within the NC16A domain (Zillikens *et al*, 1997b; Kromminga *et al*, 2002).

Noteworthy, 54% of the tested BP serum samples recognized at least an additional antigenic reactive site other than the NC16A domain. Specifically, 21 BP sera (37%) reacted with other extracellular epitopes, whereas 16 (28%) bound intracellular epitopes of BP180. These findings correlate well with recent studies showing that reactivity to BP180 is not limited to the NC16A domain, but involves additional antigenic sites within both the ICD and ECD of BP180 (Murakami *et al*, 1998; Nakatani *et al*, 1998; Egan *et al*, 1999, 2001; Nie and Hashimoto, 1999; Perriard *et al*, 1999; Schumann *et al*, 2000). Specifically, the frequency of recognition (23%) of the two novel epitopes (AA 915–985 and AA 1080–1107) in the BP180 mid-portion was similar to that previously reported for a large recombinant fragment of BP180 spanning residues 850–1171 (Nie and Hashimoto, 1999). It should be noted that recognition of these two representative epitopes was also confirmed by dot blot analysis using GST fusion proteins, demonstrating that the detected reactivity does not depend on the molecular context used. In the COOH-terminal region of the ECD of BP180, two additional overlapping epitopes (AA 1350–1387 and AA 1354–1406) were identified. These two epitopes are contained in the region spanning AA 1331–1404, which was recognized by 19% of our 57 BP serum samples, a frequency comparable to that reported for a recombinant protein consisting of residues 1365–1413 (Murakami *et al*, 1998). In the ICD, 21% of the tested BP serum samples bound to either AA 151–170 or AA 176–203, two closely located epitopes comprising less than 30 amino acids, and 10.5% of BP sera recognized the amino acid stretch 299–354. These results strongly suggest that the central portion of the ICD of BP180 is highly immunogenic. This idea is further supported by the observation that the epitope AA 151–170 was also recognized by 1A8c MoAb, indicating that this site is also immunogenic in mice.

Our results provide evidence that in BP the autoantibody response to BP180 is frequently directed to several epitopes, the pattern of which may substantially vary among patients. In this context, recent studies suggest that a distinct pattern of reactivity with BP180 is related to a distinct clinical involvement, such as disease extent or presence of mucosal lesions (Balding *et al*, 1996; Hofmann *et al*, 2002). Our findings confirm that mucosal involvement in BP is associated with a distinct epitope pattern (see Table IV). Specifically, reactivity with two extracellular epito-

pes consisting of a 34 AA stretch in the NC16A domain (AA 508–541) and a COOH-terminal 74 AA stretch (AA 1331–1404) was significantly associated with mucosal involvement, suggesting that autoantibody reactivity with multiple regions of the ECD of BP180 contributes to the development of mucosal lesions.

Findings obtained in various animal models have suggested that autoimmune diseases are characterized by an "early" phase, in which the immune response is restricted to one or two epitopes within the antigen (dominant epitopes), and a "late" phase, in which the specificity spreads to additional subdominant epitopes (epitope spreading) (Chan *et al*, 1998; Vanderlugt and Miller, 2002). Some evidence for the occurrence of an epitope spreading phenomenon has also been reported in humans (Tuohy *et al*, 1999; Bonifacio *et al*, 2000).

In BP disease, based on the results of *in vitro* studies (Sitaru *et al*, 2002), *in vivo* animal models (Hall *et al*, 1993; Liu *et al*, 1993), and clinical observations (Haase *et al*, 1998; Schmidt *et al*, 2000; Amo *et al*, 2001; Hofmann *et al*, 2002; Kobayashi *et al*, 2002), it has been speculated that autoantibodies to the NC16 subdomain are pathogenically critical, whereas development of antibodies against intracellular antigenic determinants on BP180 (and BP230) is a secondary event in the context of an "epitope spreading phenomenon" (Chan *et al*, 1998). Nevertheless, our study discloses some novel, unexpected findings. First, untreated BP patients with a disease duration of 3 mo exhibited a trend, although not significant, to display higher binding reactivity with intracellular epitopes than patients with a disease duration greater than 3 mo. Second, preliminary analysis of the epitope pattern in BP patients followed at various time points of their disease detected binding to BP-specific intracellular epitopes at an early stage of the disease in two out of five BP patients examined. Finally, it is noteworthy that in a BP patient (BP62) in whom autoantibody reactivity with NC16A domain was detectable before the development of the disease, binding to an additional intracellular epitope was found shortly after when full-blown bullous lesions developed, supporting for the first time an "intramolecular epitope spreading" phenomenon in BP.

In conclusion, by characterizing a series of epitopes of BP180 targeted by anti-BP180 autoantibodies and providing a detailed epitope pattern in a large panel of BP patients, our study provides novel insights into the autoimmune response to BP180. Our approach may be useful to characterize the autoimmune response in other autoimmune blistering disorders of the skin, such as in the pemphigus group. The developed immunologic screening procedure with BP180 epitopes on matrix constitutes a rapid diagnostic tool that appears to have a sensitivity higher than that obtained by the standard immunoblotting procedure of keratinocyte extracts. By allowing the determination of an epitope pattern that reflects the clinical phenotypes of BP, this approach might provide predictive markers relevant for management of the patients. Preliminary data on the reactivity of some BP patients against BP180 intracellular epitopes in an early stage of the disease suggest a hitherto unrecognized importance of intracellular epitopes in the initiation and development of disease. Further characterization of epitope spreading in a larger group of prospective patients is key to the understanding of the dynamic of the immune response to BP180 and, hence, of the pathogenesis of BP, and it is hoped that it will facilitate the development of specific immunologic treatments.

Results

Construction and characterization of the BP180 random epitope λ library To characterize the epitope pattern of BP serum samples, we prepared a phage λ library of BP180 random peptides fused to the D capsid protein using the λ171loxP^{-} vector. This bacteriophage contains the coding sequence for the D protein carrying at its 3′ end Spe I and Not I restriction sites, which allow subcloning of cDNA fragments of interest. The BP180 cDNA random fragments generated by DNase I digestion were ligated to adapters A and B with Spe I and Not I restriction sites, respectively, and then cloned into the λ171loxP^{-}. The library complexity, calculated as total number of independent clones, was 1.3×10^6. Control re-ligated vector yielded 5×10^3 plaques, 0.4% of the entire library, indicating that the vast majority of the phages contained an insert. Random sampling, amplification, and sequencing of 50 plaques showed that each clone contained a unique cDNA sequence between 30 bp and 700 bp, with the most represented sequences in the library being 201–300 bp long (Fig 1).

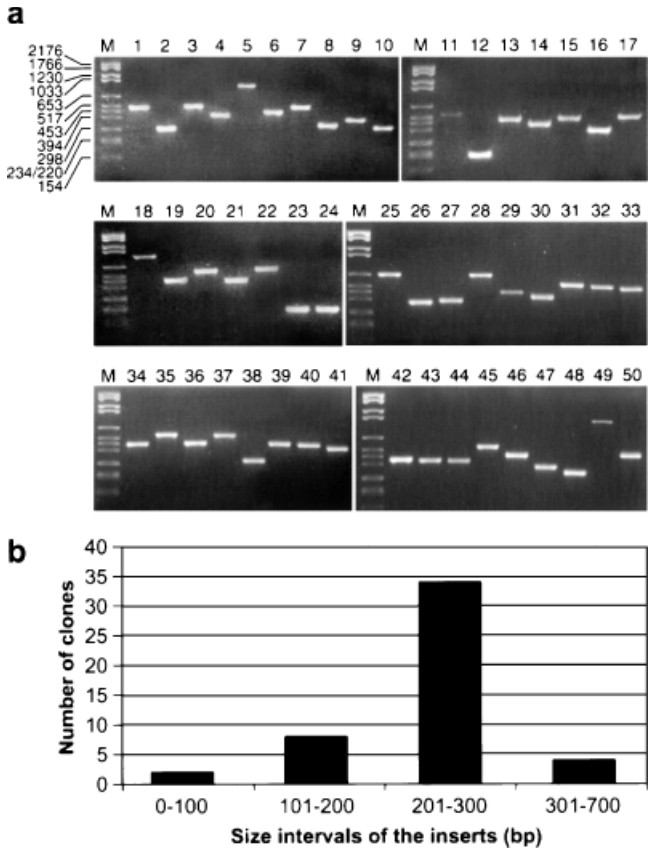

Figure 1
The inserts in the BP180 random epitope library are unique and range from 30 to 700 bp. (*a*) PCR amplification of 50 randomly selected phage clones from the library. The *inserts* are different in length and their size ranges from 30 bp to 700 bp. M, molecular weight marker. (*b*) Diagram depicting the size and distribution of the *inserts*, cloned in the bacteriophage λ vector. The length of the more represented *inserts* in the library varies between 201 and 300 bp.

Epitope mapping of three anti-BP180 MoAb To test whether the BP180 fragments are efficiently displayed on the λ capsid surface and to validate the random epitope library, we first carried out an affinity selection using the MoAb 1D1 and 233 binding to the ECD of BP180, as well as the MoAb 1A8c directed against its ICD (Hirako *et al*, 1998). Two rounds of affinity selection were performed and each time the phages were plated as plaques and probed with the same MoAb used for their enrichment. Eleven, 14, and 12 clones immunoreactive with MoAb 1A8c, 233, and 1D1, respectively, were isolated. The recognized peptide stretches were deduced on the basis of the corresponding sequence of the cDNA inserts. Alignment and comparison of the bound regions allowed us to precisely map the relevant antigenic reactive sites (Fig 2). The MoAb 1A8c bound to a nine AA stretch encompassing residues 155–163, the MoAb 233 reacted with a 26 AA stretch spanning residues 1118–1143, whereas the MoAb 1D1 detected a 31 AA stretch corresponding to residues 1357–1387 (Fig 2). In extension to previous studies (Borradori *et al*, 1997; Hirako *et al*, 1998), these findings allow us to substantially narrow the antigenic sites recognized by the three utilized MoAb and validate the random epitope library of BP180 used for screening.

Identification of BP180 epitopes by affinity selection of the random epitope library Previous studies have shown that human serum samples can be successfully employed to select epitopes from an antigen random epitope λ library (Kuwabara *et al*, 1997). Therefore, to identify BP180-specific epitopes, three rounds of biopanning of the BP180 library were carried out utilizing 10 serum samples obtained from BP patients (Table I). To isolate BP-associated phages from the library pool, we used a three-step procedure: (1) biopanning of the library with a BP serum; (2) immunologic screening of the selected pool with the same serum used in the selection; and finally (3) counter-screening with 10 NHI control sera. The 10 randomly selected BP sera (Table I) used in the affinity selections were all able to enrich specific BP180 epitopes from the library. These selections resulted in the identification of 15 epitopes spread over the entire BP180 molecule. Eight of 15 epitopes (AA 120–160, AA 151–170, AA 176–203, AA 266–343, AA 282–341, AA 299–354, AA 292–367, and AA 353–399) mapped in the ICD, and seven epitopes (AA 508–541, AA 567–622, AA 773–798, AA 915–985, AA 1080–1107, AA 1331–1404, and AA 1354–1406) were located in the ECD (Fig 3*a*). The results of the λ library

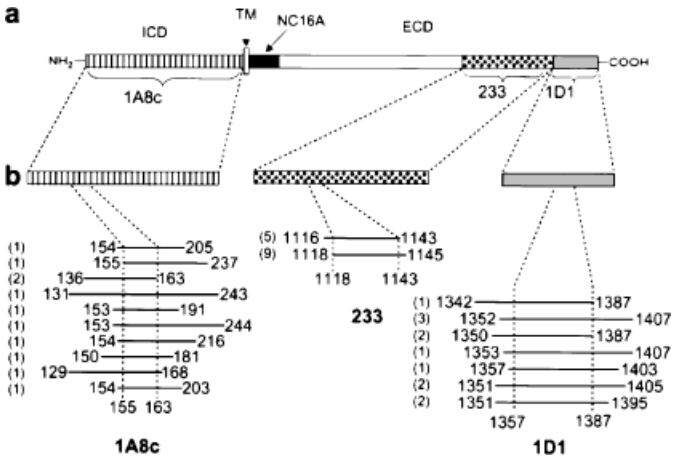

Figure 2
Mapping of the epitopes recognized by three anti-BP180 MoAb markedly narrows the previously described recognized regions. (*a*) Schematic representation of BP180: The *hatched*, *dotted*, and *shadowed* boxes represent the regions recognized by MoAb 1A8c, 233, and 1D1 as previously described (Hirako *et al*, 1998). (*b*) The previously mapped regions are aligned with the peptide sequences affinity selected with the 1A8c, 233, and 1D1 MoAb. The positions of the first and last amino acid of the selected peptides are indicated. Each sequence was independently isolated with a frequency indicated in parenthesis. The alignment allows us to define the recognized region that is shared by all the phages selected with each MoAb. TM, transmembrane domain.

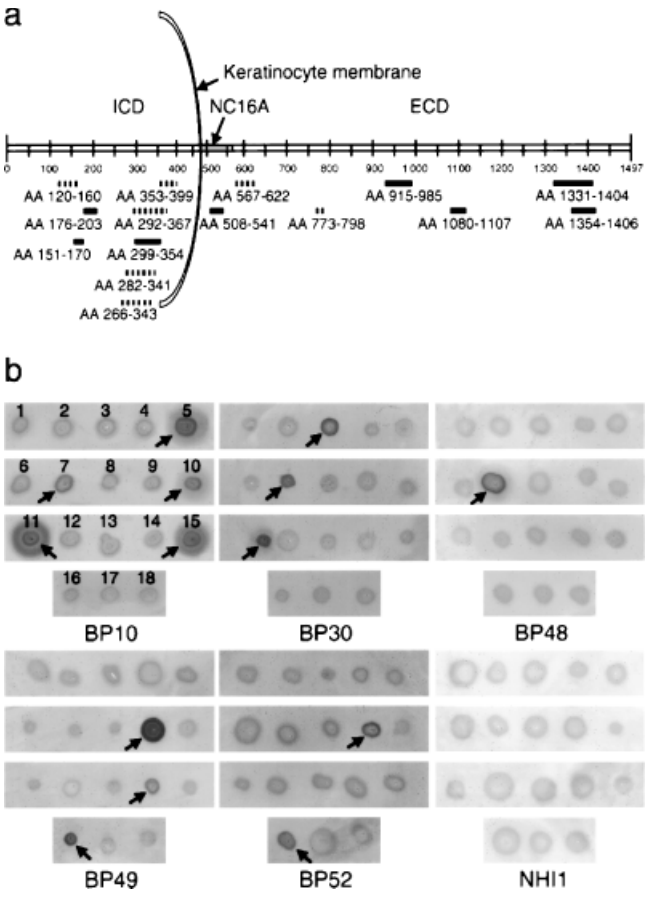

Figure 3
BP sera bind to the epitopes affinity selected from the BP180 random epitope library. (*a*) Schematic diagram representing BP180 and the position of the BP epitopes selected from the library using 10 BP sera. BP-specific epitopes, as assessed by subsequent immunoscreening with 57 BP sera and 64 NHI sera, are shown as *black lines*, whereas epitopes recognized with comparable frequency by both BP and NHI sera are depicted as *hatched lines*. For each epitope, the AA residues that define the antigenic sites are indicated. (*b*) Representative immunoscreened filters. Positive signals are indicated (*arrows*) and correspond to phages exposing BP180 peptides that were recognized by the BP sera. The position of the epitopes in the matrix is depicted on filter BP10: *1*, epitope encompassing residues 266–343 (AA 266–343); *2*, phage clone without any peptide exposed on its capsid surface; *3*, AA 567–622; *4*, AA 282–341; *5*, AA 299–354; *6*, phage clone with unrelated peptide 1; *7*, AA 508–541; *8*, AA 1080–1107; *9*, AA 1331–1404; *10*, AA 292–367; *11*, AA 176–203; *12*, phage clone with unrelated peptide 2; *13*, AA 773–798; *14*, AA 915–985; *15*, AA 353–399; *16*, AA 1354–1406; *17*, AA 120–160; *18*, AA 151–170. Control serum (NHI1) does not show any positive signal.

affinity selections demonstrate that this approach is useful and efficient to isolate BP-related epitopes, confirm that BP sera reactivity against BP180 is not restricted to the NC16A domain, and identify several novel epitopes that narrow previously described antigenic regions.

Reactivity of 57 BP sera against the selected epitopes To further characterize the BP180 epitope pattern of anti-BP180 autoantibodies, we assayed the reactivity of 57 additional BP sera, 17 pemphigus sera, and 64 NHI control sera against the 15 selected epitopes. To this purpose, we constructed a matrix that was immunoscreened with BP and control sera (Fig 3*b*). Eight of 15 epitopes showed a significantly higher frequency of reactivity with BP sera than with NHI control sera, as assessed by Fisher's exact probability test (Table III). The 17 pemphigus serum samples used as additional negative controls did not show any reactivity with these eight epitopes except for epitope AA 915–985, which was recognized by a single pemphigus serum (Table III). This finding was not unexpected, as reactivity of pemphigus sera with BP180 has been rarely reported (Schumann *et al*, 2000). Seven BP180 epitopes (Table III) were recognized with comparable frequency by both NHI and BP sera and hence were excluded from evaluation.

Thirty-four (59.6%) of the 57 tested BP serum samples recognized the stretch AA 508–541 located within the NC16A domain, a region previously shown to contain immunodominant epitopes (Zillikens *et al*, 1997b). Thirty-one of 57 (54.4%) BP serum samples, however, bound to additional antigenic reactive sites of the ICD and ECD of BP180. Specifically, whereas 21 (36.8%) BP sera recognized epitopes of the ECD of BP180 other than the NC16A, 16 (28.1%) recognized epitopes on the ICD.

Within the ECD of BP180, the epitopes AA 915–985 and AA 1080–1107, located in the mid-portion of the ECD, were recognized by eight (14.0%) and five (8.8%) BP sera, respectively (Table III). Furthermore, nine (15.8%) BP sera concomitantly recognized two overlapping COOH terminal regions of the ECD, AA 1331–1404 and AA 1354–1406 (Table III). Two additional sera, BP11 and BP44, selectively bound to epitope AA 1331–1404, but not to epitope AA 1354–1406, bringing the recognition percentage of the larger fragment to 19.3%. Noteworthy, these two sera also showed binding activity with epitope AA 1350–1387 selected using the MoAb 1D1 (data not shown). These findings indicate that BP11 and BP44 sera bind to a distinct antigenic site encompassing residues 1350–1353, which is absent from epitope AA 1354–1406 but present in epitope AA 1331–1404. Based on these results, it is very likely that the AA stretch 1331–1404 contains at least two distinct epitopes.

Within the ICD, epitopes AA 151–170, AA 176–203, and AA 299–354 were recognized by seven (12.3%), six (10.5%), and six (10.5%) BP sera, respectively (Table III). Two of the six BP sera reacting to epitope AA 299–354 failed to recognize the larger fragment AA 292–367. It is thus conceivable that the epitope in the AA 299–354 fragment is either conformational or masked in the AA 292–367 fragment. Noteworthy, the epitopes AA 151–170 and AA 1354–1406 bound by BP sera were also recognized by MoAb 1A8c and 1D1, respectively, suggesting that these regions are immunogenic in both mice and humans.

Together, 46 (80.7%) BP sera recognized at least one of the eight epitopes selected from the BP180 library (Table III). This reactivity was higher than that found against BP180 alone (61.8%) or both BP180 and BP230 (67.3%), as assessed by immunoblotting analysis of keratinocyte extracts (Table II). Six of 11 BP sera that did not react with any of the eight BP-specific epitopes were positive by immunoblotting analysis of keratinocyte extracts reacting with BP180 and/or BP230 (Table II). The remaining five BP sera might recognize an epitope not represented both in our library and in denaturated keratinocyte extracts, or even a different antigen.

To demonstrate that BP sera reacted with BP180 epitopes also in a molecular context different from the D protein of the λ capsid surface, two representative epitopes (AA 1080–1107 and AA 915–985) were then produced as GST fusion proteins. BP serum samples that recognized the selected epitopes on the phage also showed significant reactivity with GST-1080 and GST-915 in a dot blot analysis (data not shown).

Altogether our findings unequivocally demonstrate that, in addition to NC16A, several other epitopes in both the ICD and ECD of BP180 are recognized by a significant percentage of BP serum samples.

BP patients with both skin and mucous membrane involvement show reactivity with epitopes in the ECD of BP180 As previous studies have suggested that IgG autoreactivity with certain antigenic regions of BP180 is associated with peculiar clinical findings (Balding *et al*, 1996; Hofmann *et al*, 2002), we explored the potential relationship between BP180 epitope pattern and clinical features. In particular, we assessed whether our identified NC16A (AA 508–541) and C-terminus (AA 1331–1404) epitopes could be associated with mucosal lesions, as recently reported for the N- and C-terminus regions of BP180 ECD (residues 490–811 and 1351–1497, respectively) (Hofmann *et al*, 2002). The results showed a significant correlation between the presence of both skin and mucous membrane involvement and reactivity with epitopes in the ECD of BP180 ($p = 0.023$) (Table IV). Specifically, BP patients with mucosal involvement showed more frequently (three of eight; 37.5%) an autoantibody response to both the epitopes AA 508–541 and AA 1331–1404 than BP patients without mucosal involvement (two of 43; 4.7%). Although based on a limited number of BP patients these findings suggest that autoantibody reactivity with multiple regions of the ECD of BP180 relates to the development of mucosal lesions.

Finally, in the 39 serum samples obtained from BP before treatment initiation IgG reactivity with either single or grouped BP180 epitopes was not significantly related to the duration of the disease. Noteworthy, there was no trend for an increased binding activity of BP sera with intracellular epitopes in the course of the disease. In contrast, although the difference did not reach statistical significance, reactivity with intracellular epitopes appeared even higher in BP patients with a disease duration of 3 mo (10 of 20; 50%) than in those with a disease duration of more than 3 mo (four of 19; 21%) (data not shown).

BP patients show a specific epitope pattern during the disease course To evaluate the potential of our approach

Table IV. Relationship between the presence of mucosal involvement in BP patients and autoantibody response against epitopes in the extracellular region of BP180

		Mucosal involvement		
		–	+	Total
AA 1331–1404 +	–	41 (95.3%)	5 (62.5%)	46 (90.2%)
AA 508–541[a]	+	2 (4.7%)	3 (37.5%)	5 (9.8%)
Total		43 (100%)	8 (100%)	51 (100%)

[a]A BP serum was considered positive when reacted against AA 1331–1404 and AA 508–541. $p = 0.023$ as determined by Fisher's exact probability test.

in studying the BP180 epitope pattern during the course of disease, serum samples from five BP patients were collected at various disease stages and analyzed by matrix immunologic screening. The first patient, BP58, who fulfilled from the beginning the diagnostic criteria for BP, exhibited the same pattern of reactivity with both an intracellular (AA 282–343) and extracellular (AA 773–798) epitope from the early pruritic nonbullous stage to the full-blown bullous stage until therapy-induced clinical remission over a 31 mo period (Table V). Two other patients (BP59 and BP60) in the bullous phase of the disease showed reactivity with the NC16A fragment (AA 508–541), which disappeared during therapy-induced clinical remission (Table V). The remaining two patients, BP61 and BP62, were first assessed in the prodromal phase of the disease when diagnostic criteria for BP were still unfulfilled (i.e., they did not exhibit tissue-bound immune deposits along the BMZ). Whereas the BP61 serum did not show any reactivity, the BP62 serum reacted with the NC16A fragment (AA 508–541) at this early stage. When full-blown disease developed, BP62 serum, in addition to the NC16A fragment, also bound to an intracellular epitope of BP180 (AA 176–203), whereas BP61 serum recognized the NC16A fragment as well as various intracellular epitopes. The reactivity pattern of the BP61 and BP62 sera remained then stable (Table V). These preliminary results suggest that (1) BP patients show a distinct BP180 epitope profile in the course of the disease, and (2) binding to both the NC16A and intracellular epitopes can be detected early in the course of the disease.

Materials and Methods

Patients and controls Ten randomly selected BP sera were used to affinity select BP180 epitopes from the random epitope library (Table I). Serum samples, used to study the reactivity with the BP180 selected epitopes, were obtained from 57 BP patients, of whom 39 had not begun any specific treatment and 14 were treated at the time of blood draw (no information about the treatment status of the remaining four patients was available) (Table II). Forty-three of 57 BP patients presented only skin blisters without any mucous membrane involvement, and eight also had mucosal lesions in the absence of residual scarring (no information about mucous membrane involvement of the remaining six patients was available) (Table II). In addition, sera were collected from five BP patients at various time points during the disease course. The clinical diagnosis of BP was confirmed by direct immunofluorescence (DIF) assay, showing a linear deposition of IgG and/or C3 along the BMZ, and by indirect immunofluorescence (IIF) assay, showing IgG autoantibodies binding the epidermal side of 1 M NaCl split human skin (Tables I, II). Forty and 25 out of 65 serum samples recognized BP180 and BP230, respectively, by immunoblotting analyses with human keratinocyte extracts as substrate (Tables I, II). Controls included 17 pemphigus patient sera positive by DIF and IIF (autoantibodies reacting with the surface of epithelial cells of monkey esophagus) and sera from 64 normal healthy individuals (NHI) (age range 18–73 y). The study was conducted in accordance to the Declaration of Helsinki guidelines and approved by the IDI-IRCCS Ethic Committee; all patients gave informed consent.

Monoclonal antibodies and immunologic reagents Mouse IgG_1 monoclonal antibodies (MoAb) 1D1, 233, and 1A8c, which recognize the ECD (1D1 and 233) and the ICD (1A8c) of BP180 (Hirako *et al*, 1998), were used to validate the BP180 random epitope library. An alkaline phosphatase conjugated rabbit anti-mouse IgG (H + L) (Southern Biotechnology Associates, Birmingham, AL) and an alkaline phosphatase conjugated goat antihuman IgG (Fc-specific) (Sigma, St Louis, MO) were used as secondary antibodies in the immunologic screening procedure for the MoAb and BP sera epitope mapping, respectively.

BP180 random epitope λ library construction The human BP180 cDNA (Borradori *et al*, 1997) cloned in the pCDNA3 vector (Invitrogen, Carlsbad, CA) was partially digested with DNase I (Roche Diagnostics, Basel, Switzerland). The digested DNA was then fractionated by 1.5% agarose gel electrophoresis, and the fragments from 50 bp to 500 bp were excised and purified with the Wizard DNA purification system (Promega, Madison, WI). The ends of these fragments were filled in with the T_4 DNA polymerase (New England Biolabs, Beverly, MA) and ligated using 400 units of the T_4 DNA ligase (New England Biolabs) with 20-fold molar excess of adapters A and B. The double strand adapter sequences were as follows: A, forward 5′-CTAGTGGCAGTGGTAGCGGC-3′; reverse 5′-GCCGCTACCACTGCCA-3′, and B, forward 5′-GCTGGTTTCA-AGTGC-3′; reverse 5′-GGCCGCACTTGAAACCAGC-3.

The 16-mer and 15-mer oligos from adapters A and B, respectively, were 5′ phosphorylated. Unligated and self-ligated adapters were removed by separation with 2% agarose gel electrophoresis and fragments ligated to adapters were purified with the Wizard DNA purification system (Promega). The purified DNA fragments were ligated with the vector λ171loxP$^-$ (Santi *et al*, 2000) (kindly provided by Dr A. Nicosia, IRBM, Pomezia, Italy), digested with Spe I and Not I restriction enzymes. The ligation mixture was then packaged using λ packaging kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. The resultant phages were plated at 5×10^5 plaque-forming units (pfu) per 150 mm plate on *Escherichia coli* strain BB4 (Stratagene, La Jolla, CA) and prepared as a lysate (Sambrook *et al*, 1989).

Biopanning procedure The BP sera autoantibodies and the MoAb epitope mapping were performed by coating 60 mm diameter polystyrene dishes overnight at 4°C with 1 μg per mL Protein A (Amersham Biosciences) and 1 μg per mL rabbit antimouse immunoglobulins (Dako, Carpinteria, CA), respectively,

in 2 mL of coating buffer (50 mM $NaHCO_3$ pH 9). After two washes with blocking solution (3% bovine serum albumin, 1% Triton in 1 × phosphate-buffered saline (PBS)), the dishes were blocked with 10 mL of the same solution for 2 h at 37°C. BP sera (10 μL) or hybridoma supernatants of the MoAb (10–40 μL) were preincubated with gentle agitation for 30 min at 37°C in 1 mL blocking solution with 10 mM $MgSO_4$ and 10 μL of BB4 bacterial extracts. One hundred microliters of BP180 random epitope λ library (about 10^{10} pfu) were then added to the preincubation mixture and incubation was carried out for 1 h at 37°C. This mixture was placed on the Protein-A- or antimouse-immunoglobulin-coated dishes for 30 min at room temperature under gentle agitation. Unbound phages were removed by extensive washing with 1% Triton, 10 mM $MgSO_4$ in 1 × PBS. Bound phages were eluted by incubating the dish with BB4 cells for 20 min at room temperature. Cells infected by phages were recovered and used for phage titration and immunologic screening. The phages were then affinity selected for two or three cycles (rounds).

Immunologic screening Phage plaques grown on BB4 cells were blotted onto a nitrocellulose filter (Millipore, Bedford, MA). The filter was washed three times for 10 min at room temperature with washing solution (0.1% Triton in 1 × PBS) and incubated with blocking solution (5% skimmed milk, 0.1% Nonidet P-40 in 1 × PBS) for 3 h at room temperature. Each BP serum (10 μL) was preincubated with gentle agitation for 30 min at 37°C in 4 mL of blocking solution with 25 μL per mL of BB4 bacterial extracts and 3.5 × 10^9 pfu per mL of λ control phage (λ171loxP$^-$). This mixture was added to the nitrocellulose filter and incubated under gentle agitation overnight at 4°C. Then, the filter was washed and incubated with the appropriate alkaline phosphatase conjugated secondary antibody for 1 h at room temperature. The filter was washed eight times and then stained with 330 μg per mL of nitroblue tetrazolium (NBT) and 165 μg per mL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche Diagnostics) in substrate buffer (100 mM NaCl, 100 mM Tris−HCl pH 9.6, 5 mM $MgSO_4$). The DNA coding sequences of the epitopes exposed on phage clones exhibiting positive immunostaining were amplified by PCR. The forward primer 5′-ACGTTCCGTTATGAGGATGT-3′ and reverse primer 5′-CCACTACGTGAACCATCAC-3′ were annealed upstream and downstream of the insertion site of BP180 fragments in the λ171loxP$^-$. The PCR cycle profile was 1 min at 94°C, 1 min at 58°C, 1 min at 72°C for 30 cycles, followed by an elongation step of 10 min at 72°C. The amplified products were purified with the High Pure PCR product purification kit (Roche Diagnostics) sequenced using the BigDye terminator cycle sequencing kit and run on an automated sequencer ABI prism 377 (Applied Biosystems, Roche Molecular Systems, Branchburg, NJ).

Matrix construction All positive phages displaying a BP180 epitope on their capsid surface were amplified by preparing a phage lysate. Phages were peaked as a plaque on NZY plate with BB4 starved cells poured with top agar and incubated overnight at 37°C. The lysis spots obtained were blotted onto nitrocellulose filter and immunoscreened with BP or control sera.

The immunologic screening procedure was performed as described above using BP serum samples diluted 1:400 in blocking buffer and an alkaline phosphatase conjugated goat antihuman IgG (Fc-specific) (Sigma) diluted 1:2000 as secondary antibody. A wild-type phage with no peptide exposed on its capsid surface and two different phage clones displaying unrelated peptides were always used as internal negative controls. The results were confirmed by performing two independent experiments.

Statistical analyses The Fisher's exact probability test was used to compare the frequencies of BP sera and NHI sera reactivity to BP180 epitopes and to assess the relationship between clinical findings and epitope pattern. A p value $\leqslant 0.05$ was considered significant.

Fusion protein production and dot blot analysis Two cDNA fragments encoding the amino acids (AA) 1080–1107 and 915–985 of human BP180 were amplified by PCR using the BP180 cDNA as template. The fragment AA 1080–1107 was amplified with the sense primer 5′-CGGGATCCCCTCCTCCATCTCTTC-3′ and antisense primer 5′-GGAATTCTCAAGGGCCCATCAAGTAC-3′. The fragment AA 915–985 was amplified with the sense primer 5′-CGGGATCCCCGGTCCCAAG-3′ and antisense primer 5′-GGAATTCTCATGATCCCCCTTCAG-3′. The underlined sequences represent the BamH I and EcoR I restriction sites. After BamH I and EcoR I digestion, the fragments were cloned into pGEX-3X (Amersham Biosciences) in order to obtain glutathione S-transferase (GST) -1080 and -915 fusion proteins. Recombinant clones were expressed in the *E. coli* strain DH5α (Invitrogen) and purified by affinity chromatography using Glutathione-Sepharose 4B (Amersham Biosciences).

GST (600 ng) and molar equivalent amounts of GST-1080 or GST-915 were dot blotted onto nitrocellulose membrane Hybond-C (Amersham Biosciences). Filters were incubated, first with blocking solution for 1 h at room temperature, and then overnight at 4°C with 30 μg per mL GST, 100 μL per mL of DH5α bacterial extracts, and 1:100 dilution of each tested serum. After washing, the filters were incubated with the appropriate alkaline phosphatase conjugated secondary antibody for 1 h at room temperature and developed with NBT and BCIP (Roche Diagnostics).

We thank A. Nicosia from IRBM for providing λ171loxP$^-$ vector and A. Sonnenberg for BP180 cDNA. We are grateful to F. Felici and p. Monaci for critical suggestions. We also thank M. Inzillo for help with the artwork and C. Failla for critical reading of the manuscript. This work was supported by grants from the European Community (QLG1-CT-2001-02007) and the Ministero della Salute, Italy.

DOI: 10.1046/j.0022-202X.2003.22126.x

Manuscript received June 20, 2003; revised September 9, 2003; accepted for publication September 16, 2003

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